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Bacterial degradation of toluene, chlorobenzene and trichloroethylene

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Mars, A. E. (1998). Bacterial degradation of toluene, chlorobenzene and trichloroethylene. Groningen: s.n.

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Download date: 12-11-2019

Summary

MICROBIAL DEGRADATION OF ENVIRONMENTAL POLLUTANTS

The pollution of ecosystems with man-made chemicals causes great concern because of the serious ecotoxic effects that many of these chemicals have. During the last two decades it became clear that microorganisms can possess enzyme systems that are able to convert environmental pollutants, and the application of such microorganisms seems to be a cost effective way for the removal of these chemicals from the environment. Nevertheless, chemicals that are biodegradable can be very persistent in ecosystems. Such recalcitrance can be caused by unfavourable environmental conditions that inhibit the desired microbial activity. Besides, chemicals might persist biodegradation when the conversion of pollutants has a negative effect on the microorganisms that degrade them. This can, for example, occur when the conversion leads to the generation of harmful reaction products, or to the accumulation of dead-end metabolites in the cells. Especially when microorganisms convert contaminants cometabolically without any gain of energy, these conversions might be very toxic.

The work that is described in this thesis deals with the inhibitory effects of cometabolic conversions of some important environmental pollutants. It aims to provide insight in the negative effects that the conversion of a cosubstrate can have on microorganisms and their biodegrading capacity, and in molecular changes that allow microorganisms to degrade mixtures of pollutants that are known to inhibit the metabolic pathways of the individual compounds.

COMETABOLIC CONVERSION OF TRICHLOROETHYLENE BY *Burkholderia cepacia* G4 UNDER GROWTH-LIMITED CONDITIONS

One of the most important contaminants of groundwater is trichlororethylene (TCE). No microorganisms are known that are able to grow with this compound as a source of carbon and energy, but cometabolic degradation of TCE is possible by microorganisms that possess aspecific oxygenases that are involved in the degradation of substrates like toluene, phenol, methane, or ammonia (e.g. Arciera et al., 1989; Leahy et al., 1996; Nelson et al., 1987; Oldenhuis et al., 1989b; Tsien et al., 1989; Wackett and Gibson, 1988). The oxygenases convert TCE to reactive oxidation products that spontaneously decompose to compounds like formic and glyoxylic acid, carbon monoxide, and chloride ions (Fox et al., 1990; Li and Wackett, 1992; Newman and Wackett, 1997). The oxidation products of TCE can react with cell components. This can lead to the inactivation of the TCE-degrading capacity of the microorganism and even to cell death (Oldenhuis et al., 1991; van Hylckama Vlieg et al., 1997; Wackett and Householder, 1989).

The sensitivities of TCE-degrading organisms to the toxic effects of TCE conversion show large differences, and the kinetic parameters of the individual strains vary as well. One of the most suitable microorganisms for the cometabolic degradation of TCE is *B. cepacia* G4. This organism converts TCE when it grows on aromatic compounds like toluene or phenol, and it combines a relatively high resistance to the oxidation products of TCE with good kinetic parameters for the degradation of this cosubstrate at lower TCE concentrations (Folsom et al., 1990; Landa et al., 1994a).

When organisms like *B. cepacia* G4 are applied in in situ bioremediation processes or in biofilms, the organisms will be confronted

with very low concentrations of growth substrates. This implicates that the bacteria must be able to convert TCE under these conditions. Moreover, they should be able to maintain themselves in the microbial population. For this, they have to compete with other bacteria for growth-limiting substrates.

There is little information about the behavior of *B. cepacia* G4 under these circumstances. Therefore, the aim of the work that is described in Chapters 2 and 3 is to obtain more insight in the effects of TCE degradation on this strain under severe substrate-limiting conditions.

In Chapter 2 it was described that *B. cepacia* G4 degrades 65% of the incoming of TCE at a rate of 26.5 nmol mg of cells (dry weight)$^{-1}$ h^{-1} when the organism is cultivated in a non-growing fed-batch culture at a toluene/TCE ratio of 2.3. Compared with toluene only, the conversion of TCE caused a fourfold increase in the maintenance requirements of the culture from 22 to 94 nmol of toluene mg cells (dry weight)$^{-1}$ h^{-1}. This large increase is most likely caused by toxic effects that result from the conversion of TCE. When TCE was added to the culture while toluene was omitted, mutants arose which could no longer degrade toluene or TCE because they had lost the plasmid pTOM that encodes the genetic information for this degradation. These results show that *B. cepacia* G4 can degrade TCE as long as toluene is present. However, the negative effects of the conversion of TCE result in a large increase of the maintenance energy demand of the organism and the loss of the TCE-degrading capacity in the absence of primary substrate.

To determine if the toxic effects of the conversion of TCE would influence the capacity of *B. cepacia* G4 to compete with other bacteria for a growth-limiting primary substrate, the organism was cultivated together with three other toluene degraders (*P. putida* mt-2, *P. putida* F1, and *P. putida* GJ31) in a fed-batch culture at a very low toluene concentration (Chapter 3). Besides *B. cepacia* G4, the *P. putida* strains F1 and GJ31 are able to degrade TCE, although at very different rates. When the four strains were cultivated on toluene in the absence of TCE, they were all maintained at a rather constant viability, even though the kinetic parameters for growth on toluene significantly differed. However, when TCE was added to the culture, the three TCE degraders disappeared from the fed-batch culture at similar rates, and hardly any TCE was degraded. Moreover, the competitive capacity of the surviving strain (*P. putida* mt-2) was further improved by mutations that allowed this organism to grow faster on toluene, but also resulted in the loss of the capacity of this strain to grow on *p*-xylene. The results indicate that the conversion of TCE has a negative influence on the competitive capacity of the toluene-converting strains that transform TCE and can lead to the loss of the TCE-degrading capacity under non-sterile conditions.

SIMULTANEOUS DEGRADATION OF CHLORINATED AND METHYLATED AROMATICS BY *P. putida* GJ31

Besides the harmful effects of the conversion reactions that might be needed for the biodegradation of a contaminant, the presence of other pollutants can negatively influence the degradation of a compound, for example when cometabolic conversions of substrate analogues generate toxic effects. This has been observed for combinations of methylated and chlorinated aromatic compounds (e.g. Knackmuss, 1981). In general, chlorinated aromatics are converted to chlorinated catechols that are subsequently oxidized at the *ortho* position by specialized catechol 1,2-dioxygenases. The chlorine substituents are removed further down in this so-called modified *ortho*-cleavage pathway (Schlömann, 1994). Several of these compounds can serve as a growth substrate, and bioremediation of such contaminants should be feasible.

When methylated aromatic compounds are degraded, methylated catechols are usually formed as intermediates which are further

oxidized at the *meta* position by catechol 2,3-dioxygenases. Such enzymes are found in well-known toluene degraders like *B. cepacia* G4 and *P. putida* mt-2. Further degradation occurs via other enzymes of this *meta*-cleavage pathway (Smith, 1990).

The two degradation routes are often incompatible. When *meta*-cleaving enzymes are confronted with catechols that are chlorinated at the 3-position, they transform them to reactive acylchlorides that are believed to be responsible for the rapid inactivation of the enzyme and the destruction of the *meta*-cleavage activity (e.g. Bartels et al., 1984). On the other hand, methylated catechols can be converted by *ortho*-cleavage pathways to methyllactones, which have been shown to accumulate as dead-end products (Catelani et al., 1971; Knackmuss et al., 1976).

A strain (*Pseudomonas putida* GJ31) was previously isolated which is able to resist these kind of toxic effects since it can simultaneously grow on chlorobenzene and toluene (Oldenhuis et al., 1989a). The presence of toluene stimulates the degradation of chlorobenzene by *P. putida* GJ31 (Keuning and Jager, 1994). This was not the case with *Pseudomonas* sp. strain JS6, which also grows on both compounds (Pettigrew et al., 1991). The latter strain uses a *meta*-cleavage pathway when it grows on toluene, but it uses a modified *ortho*-cleavage pathway for growth on toluene in the presence of chlorobenzene. This was shown to be less efficient (Pettigrew et al., 1991).

Because organisms that can simultaneously degrade mixtures of chlorinated and methylated aromatics can have important practical applications, and *P. putida* GJ31 possesses good kinetic parameters for the degradation of these substrates (Keuning and Jager, 1994). The metabolic pathway that is used by this strain for the simultaneous growth on chlorobenzene and toluene was characterized (Chapter 4 and 5).

In Chapter 4 is described that *P. putida* GJ31 only uses a *meta*-cleavage pathway for the degradation of chlorobenzene. This is in contrast to all other chlorobenzenes-degrading microorganisms that have been described so far, since they use modified *ortho*-cleavage pathways for this (e.g. Haigler et al. 1992; Pettigrew et al., 1991; Reineke and Knackmuss, 1984; van der Meer et al., 1991b). The presence of a *meta*-cleavage pathway enables *P. putida* GJ31 to use this more efficient pathway for the degradation of toluene in the presence of chlorobenzene. Toluene and chlorobenzene are converted by a dioxygenase and a dehydrogenase to 3-methylcatechol and 3-chlorocatechol, respectively. Dehalogenation of 3-chlorocatechol occurs when the aromatic ring is cleaved by the catechol 2,3-dioxygenase of the *meta*-cleavage pathway, which leads to the formation of 2-hydroxymuconate. This substrate can be converted by the other enzymes of the *meta*-cleavage pathway. The catechol 2,3-dioxygenase of *P. putida* GJ31 is thus able to productively degrade 3-chlorocatechol without the suicide inactivation that is usually observed when these type of enzymes convert 3-chlorinated catechols.

Chapter 5 describes the cloning and sequence analysis of the catechol 2,3-dioxygenase (CbzE) of *P. putida* GJ31. The enzyme is most homologous to catechol 2,3-dioxygenases of the 2.C subfamily of the type 1 extradiol dioxygenases (Eltis and Bolin, 1996), which contains enzymes that are up to 72% identical. Three of the mostly related catechol 2,3-dioxygenases were tested for their capacity to convert 3-chlorocatechol, and they were all rapidly inactivated by this substrate. This means that CbzE is probably the only enzyme of this group that productively convert 3-chlorocatechol. Hybrid enzymes that were made of CbzE and the most similar catechol 2,3-dioxygenase TdnC showed that this capacity is determined by the C-terminal part of the enzyme. CbzE also seems to have an increased resistance to suicide inactivation with methylated catechols, which might indicate that the inactivation mechanisms are similar.